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Advances in analytical methodology for bioinorganic speciation analysis: metallomics, metalloproteomics and heteroatom-tagged proteomics and metabolomics

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The recent developments in analytical techniques capable of providing information on the identity and quantity of heteroatom-containing biomolecules are critically discussed. Particular attention is paid to the emerging areas of bioinorganic analysis including: (*i*) a comprehensive analysis of the entirety of metal and metalloid species within a cell or tissue type (*metallomics*), (*ii*) the study of the part of the metallome involving the protein ligands (*metalloproteomics*), and (*iii*) the use of a heteroelement, naturally present in a protein or introduced in a tag added by means of derivatisation, for the spotting and quantification of proteins (*heteroatom-tagged proteomics*). Inductively coupled plasma mass spectrometry (ICP MS), used as detector in chromatography and electrophoresis, and supported by electrospray and MALDI MS, appears as the linchpin analytical technique for these emerging areas. This review focuses on the recent advances in ICP MS in biological speciation analysis including sensitive detection of non-metals, especially of sulfur and phosphorus, couplings to capillary and nanoflow HPLC and capillary electrophoresis, laser ablation ICP MS detection of proteins in gel electrophoresis, and isotope dilution quantification of biomolecules. The paper can be considered as a followup of a previous review by the author on a similar topic (ref. 1: J. Szpunar, Analyst, 2000, **125**, 963).

1. Introduction

The systematic acquisition of information relevant to genomes, gene transcripts, proteins and their functions and its global and comprehensive analysis, often referred to as genomics and proteomics, is a fundamental contribution of analytical chemistry to the progress of research in life sciences.[2–5] In particular, most of the developments in the area of protein research would not have been possible without the invention of the soft ionization techniques such as electrospray[6] and MALDI[7,8] allowing the transfer of ionized large biomolecules into the gas phase, and the related advances in mass spectrometry.

The function of many proteins, referred to as metalloproteins, critically depends on their interaction with a metal, usually a transition one, such as *e.g.* Cu, Fe, Zn or Mo. There are proteins, such as *e.g.* metallothioneins, which are expressed as a defense mechanism of an organism against heavy metal stress, and others which serve within an organism as transporters of essential nutrient ions, contaminants and metal probes. The activity of intracellular metal ions is controlled by several families of proteins, either detoxifying, protecting or simply involved in cell cycle, proliferation and apoptosis.[9] The molecular bases of many of the metal-dependent biochemical processes remain elusive. The mechanisms by which the metal is sensed, stored or incorporated as a cofactor in a cell are seldom known.[10] Their understanding requires not only the identification of the proteins involved and characterization of their complexes with metals but also a characterization of the pool of non-proteinaceous molecules, of a relatively small size, some of which deliver metals to metalloproteins.[11] This pool also serves as a sink for metal-binding metabolism products of enzymatic or biochemical reactions. The analysis of metal-complexes with biomolecules at the trace levels poses a number of challenges to the analyst.[12]

Curiously, molecular mass spectrometry alone seems to be unable to provide answers to many of these vital questions. The information on the metal–protein interactions is often either lost in the gas-phase (MALDI) or not acquired because of the insufficient detection limits in real-world salt-rich

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matrices (ES MS). Also, the ionization efficiency is dependent on the molecule which, while being of little consequence for structural analysis, renders the quantification without external standardization very difficult if not impossible. Since standards for most biomolecules of natural origin are unavailable, quantification is possible only *via* tagging, *e.g.* with an isotope-coded affinity tag (ICAT).[13] The relatively complex chemistry of the approach and the poor linearity range of ESI MS and MALDI MS often make the analytical precision and accuracy insufficient to unravel the fine differences in expressed quantities of particular proteins between the control and disease states.

All these reasons encourage a closer look to be taken at the potential role of the plasma source (elemental) MS in bioanalytical chemistry in general and in proteomics in particular.[14–16] Inductively coupled plasma mass spectrometry (ICP MS) is widely appreciated for its isotope specificity, versatility (all but a few elements are readily detectable) and high sensitivity (down to sub-femtogram levels).[17] It becomes a molecule-specific technique when applied as a detector in chromatography or electrophoresis. Its unmatched advantages include a large (10^5–10^6) linear dynamic range and the virtual independence of the signal intensity of the structure of the biolomolecule and the matrix.[17]

Since its introduction in the 80s for element specific detection, ICP MS has been widely used for trace element speciation studies and dethroned other element-specific techniques such as atomic absorption, emission or fluorescence spectrometry.[18] The overwhelming majority of applications concerning probing and quantification of metalloproteins were developed using ICP MS coupled to size-exclusion chromatography. More selective separation mechanisms, such as ion-exchange or reversed-phase were mostly reported for non-protein biomolecules, *e.g.* organoselenium or organoarsenic compounds. Because of the poor separation from the matrix components, attempts by molecular MS to identify the metallobiomolecules detected by ICP MS at the picogram level often failed. These approaches were comprehensively reviewed.[1,15,19]

Recent years have witnessed a number of interesting developments both in the area of the interfacing of the well established high-resolution techniques for separation of biomolecules, such as gel electrophoresis or capillary HPLC, to ICP MS, and in the field of interference-free high-sensitive detection of the biologically vital elements, such as sulfur and phosphorus. They make ICP MS an attractive partner of electrospray and MALDI MS for the investigation of metallobiomolecules in complex biological matrices. The number of reports of approaches showing the synergy between ICP MS and ES MS/MS is growing exponentially.[16,20]

The emerging areas of relevant applications include analysis for metal binding proteins, either by virtue of being metalloproteins or having metal-binding sites. More generally, the accurate quantification of peptides and proteins can be accomplished *via* a covalently bound ICP MS-detectable heteroatom, either already present (S, P, Se) or added as a tag (any metal). A field of metallomics: a global analysis of the entirety of metal and metalloid species with a cell or tissue type is emerging as one of the most dynamic research areas in trace element speciation analysis.[21–23] Structural biology and genomic studies require high-throughput methods to screen proteins for structure and function, and one-third of proteins are critically dependent of the presence of a metal.[24]

This critical review reflects the evolution of hyphenated techniques for bioinorganic speciation analysis during the last 5 years. It focuses, on one hand, on a number of proteomics-related applications which have particularly benefited from elemental mass spectrometry and, on the other hand, on advances in the synergic use of ICP MS, ES MS/MS and MALDI MS for obtaining species-specific information on metallobiomolecules in biological systems. A low profile is kept on approaches, referred to as Afractionation@,[1,15,25] in which the purity of the species arriving at the detector is not satisfactorily demonstrated. The historical evolution and fundamentals of analytical techniques discussed here are omitted on purpose and the reader is invited to consult the previous text of the author on this topic.[1] The citations considered are basically peer reviewed publications not older than 2000.

2. Terms and concepts

In order to function an organism must regulate a proper assimilation and incorporation of trace metals. Therefore, the chemistry of a cell needs to be characterized not only by its characteristic genome in the nucleus and a protein content, a proteome, but also by the distribution of the metals and metalloids among the different species and cell compartments, *metallome*. The latter term would therefore refer to the entirety of individual metal species in a cell and encompass the inorganic (free and complexed) element content and the ensemble of its complexes with biomolecules, and especially with proteins, metalloproteome. This definition of the metallome: the entirety of metal and metalloid species within a cell or tissue type, seems to be a much better analogy with genome and proteome than the reference to the "free metal content of a cell" only.[21]

The definition of metallome should be seen in the context of speciation of an element, defined by IUPAC as the distribution of an element amongst defined chemical species in a system.[25] Speciation analysis, *i.e.* analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample, narrowed to metallobiomolecules, can be referred to as metallomics, per analogy to genomics and proteomics. In other words, metallomics can be considered as a subset (referring to cellular biochemistry) of speciation analysis understood as the identification and/or quantification of elemental species. Species of interest for metallomics will include complexes of trace elements and their compounds (*e.g.* metal probes) with endogenous or bio-induced biomolecules such as organic acids, proteins, sugars or DNA fragments. Deciphering a metallome will thus inform us of: (i) how an element (metal or metalloid) is distributed among the cellular compartments of a given cell type, (ii) its coordination environment; in which biomolecule it is incorporated or by which bioligand it is complexed, and (iii) the concentrations of the individual metal species present. Monitoring the changes of the metallome as a function of time and exposure to external stimuli can be referred to as comparative metallomics.

The analytical approach based on hyphenated techniques using the parallel ICP MS and ES MS/MS detection is also increasingly used for the analysis of proteins which do not contain a metal or metalloid but another heteroelement, such as *e.g.* sulfur, of which the presence is pivotal to the analytical strategy employed. Also, a heteroelement may be added to the protein by way of derivatization as an external tag. For these applications a term "heteroatom-tagged proteomics" was proposed,[26] whereas the tagging element can be either already present or introduced by means of derivatization.

Table 1 summarizes definitions of terms used in the area of bioinorganic analytical chemistry.

3. Bioinorganic speciation analysis: analytical targets

Bioinorganic chemistry is a rapidly expanding discipline due to recent advances in inorganic spectroscopy, genetics, molecular biology and structural biology. The most important classes of compounds of interest include, on one hand, peptides and proteins, their complexes with metals, and metal-binding enzymatic metabolites, and, on the other hand, products of the metabolism of arsenic, phosphorus, or selenium leading to the formation of the covalent C–As, C–P or C–Se bonds.[1,14] There is increasing interest in studies of the metabolism of heteroatom containing drugs and metal probes whereas reports on metal complexes with other vital biomolecules such as carbohydrates and DNA-fragments remains scarce, likely because of the analytical difficulties.

3.1. Protein research

The availability of the complete sequence of an increasing number of genomes fuels developments in proteomics which are achieved usually by electrospray and MALDI mass spectrometry. Most proteins, however, can be very sensitively detected by ICP MS owing to the presence of an ICP ionizable element such as *e.g.* sulfur, phosphorus, iodine or metals. This technique, explored for *ca.* 20 years for the chromatographic detection of metal-complexes with proteins, starts being routinely used for quantification of proteins *via* the sulfur atom[26] or for quantification of post-translational modifications, such as *e.g.* phoshorylation.[27] The isotopic specificity of ICP MS encourages studies aimed at the identification of molecules acting as metal donors to various enzymes.[28]

ICP MS detectable elements in proteins and protein complexes can be of multifarious origin (Fig. 1). A number of amino acids of which proteins are built contain a covalently incorporated heteroelement, usually sulfur, selenium or iodine. A heteroelement can be introduced in a covalent way naturally, *e.g.* by post-translational phosphorylation, or artificially by means of chemical derivatization. Metals or metal-containing molecules (*e.g.* vitamin B_{12}) are used by many enzymes as cofactors. Finally, a number of amino acids have complexing functions able to bind metal ions or metal-containing molecules (*e.g. cis*-platin).

3.1.1. Proteins incorporating a heteroelement-containing amino acid. *Sulfur-containing amino acids.* The highest potential interest is enjoyed by sulfur which is present in two fairly popular amino acids, methionine and cysteine.[26] They both occur with a cumulative abundance of about 5% in natural peptides and proteins. Hence, statistically speaking, at least one sulfur-containing amino acid is expected to occur in every polypeptide exceeding a length of 20 amino acids. Therefore, the direct determination of sulfur by elemental MS offers potentially a way for quantification of peptides and proteins. Note that in the past, ICP AES was extensively used for the quantification of metallothioneins *via* determination of sulfur in HPLC.[29] However, the relatively poor sensitivity of that technique prevented wider applications.

Table 1 Terms and concepts in bioinorganic speciation analysis

Genome	The set of genes of a given organism
Genomics	Study of the genome of an organism
Ionome	(free) Metal content a cell
Proteome	The entire protein complement of a given genome, that is, the entirety of the proteins that are expressed by the genome
Proteomics	Study of the proteome of an organism. The term is most commonly associated with the use of MS to identifiy proteins expressed in a given cell type or tissue under a given set of conditions
Hetero-atom tagged proteomics	Study of the proteome in which analytical information is acquired by elemental MS either owing to the presence of heteroatom (S, Se, P, I) in a protein or introduced *via* tagging (derivatization)
Metabolome	The set of metabolites produced as a result of reactions catalysed by certain proteins (enzymes)
Metallome	The entirety of metal and metalloid species within a cell or tissue type. It encompasses, among others, the inorganic species (ionome) and protein complexes (metalloproteome)
Metallomics	Study (qualitative and/or quantitative) of the metallome
Metalloproteome	The entirety of metal complexes with proteins in a sample. Note that this term had originally been used in a narrower sense and concerned the proteins with enzymatic fuctions only
Phosphoproteome	The part of the proteome modified by phorphorylation (a post-translational modification)
Selenoproteome	The part of the proteome incorporating selenoamino acids, selenomethionine and selenocysteine. The use of the term is often limited to proteins with genetically encoded selenocysteine only
Metallo, phospho-, selenoproteomics	Analysis of a particular sub-proteome
Speciation	The distribution of an element amongst defined chemical species in a system
Speciation analysis	Analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample
Fractionation	The classification of an analyte or a group of analytes from a certain sample according to physical or chemical properties. The term often refers to low resolution chromatography where metal containing fractions are detected by AAS or ICP AES/MS

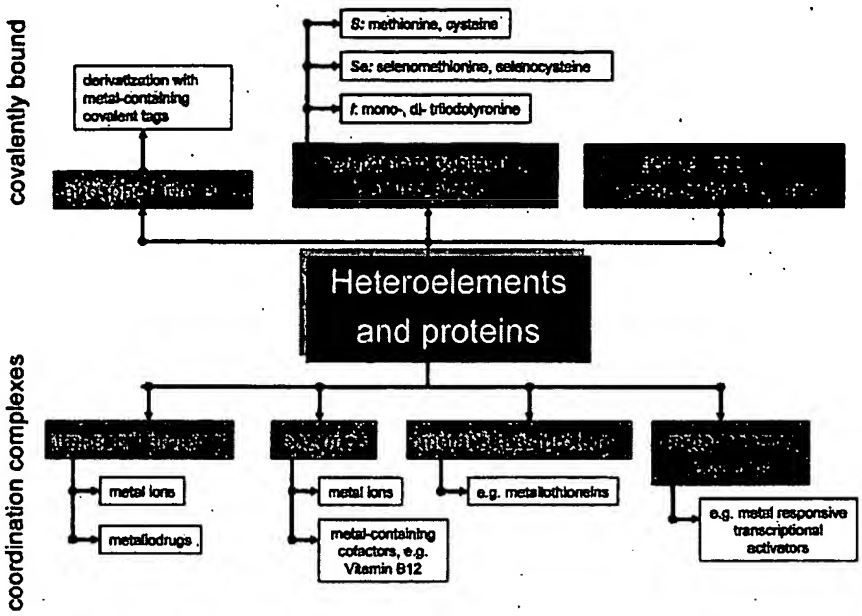

Fig. 1 Origins of heteroatoms in proteins and their complexes.

Selenium-containing amino acids. Selenium is a constituent of two major amino acids, selenomethionine and selenocysteine, which are the analogues of methionine and cysteine, respectively. Selenomethionine is believed to be deposited in proteins unspecifically in place of methionine at any position occupied by the latter. Selenocysteine (21st amino acid) is genetically encoded (UGA codon) and occurs at specific positions at the amino acid chain.[30] The selenocysteine-containing proteins are referred to as selenoproteins in contrast to selenium-containing proteins which contain selenomethionine. Selenium-containing proteins are characteristic of plants whereas selenoproteins occur in animals and humans. The most widely known selenoproteins include selenoprotein P, glutathione peroxidase and thyroglobin deiodinase. Altogether 22 mammalian selenoproteins have been found to date.[31] Using computational methods which rely on identification of selenocysteine insertion RNA structures, the coding potential of UGA codons, and the presence of cysteine-containing homologues, Kryukov *et al.* predicted that the human selenoproteome would consist of 25 selenoproteins.[32,33]

Iodine is utilised by the thyroid gland for the biosynthesis of the thyroid hormones, *e.g.* 3,3′, 5,5′-tetraiodothyronine (T4) and 3,3′,5-triiodothyronine (T3). They both are constituents of thyroglobuline and a specific detection of iodine may be a method for quantification of this protein.[34]

3.1.2. Phosphoproteins. Phosphorylation on serine, threonine and tyrosine residues is an important modulator of protein functions.[35] Organisms use reversible phosphorylation of proteins to control many cellular processes including signal transduction, gene expression, the cell cycle, cytoskeletal regulation and apoptosis. Mass spectrometry, both MALDI and ESI MS, have been efficiently used for the identification of phosphorylation structures in proteins in conjunction with, and more often in replacement of, the classical methods using Edman sequencing and ^{32}P-phosphopeptide mapping for localization of phosphorylation sites.[35,36]

The major problems in phosphoproteomics are due to the generally very low stoichiometry of phosphorylation, the limited dynamic range allowing an easy location of the major phosphorylation sites but not of the minor ones, and the difficulties with quantification.[36] Indeed, the most effective MS approach, based on tracing the *m/z* 79 ion yielded by CID of the phosphorylated peptides during an LC MS/MS analysis of the digested protein, does not provide vital information of the number of phosphorylated sites per peptide, nor the absolute quantification.[27] Hence the interest in the complementary information by ICP MS on the absolute quantity of phosphorus eluting in each peak.[37]

3.1.3. Metal-binding proteins. Metal–protein interactions are important for the functions of many proteins and therapeutic drugs. Numerous essential biological functions require metal ions, and most of these metalloproteins. The incorporation of the metal ion is a very tightly regulated process that, *in vivo*, often requires specific chaperones to deliver and help incorporate the metal atom in the macromolecule.

Originally, metalloproteins, in the proper sense of the term, were considered distinct from metal–protein complexes.[38] Whereas the former featured high-affinity interactions that were not lost during the isolation step, the latter's interactions were lower affinity and were easily lost during sample handling. However, it is clear that a continuum exists of biologically relevant metal ion binding to proteins.[38] Therefore, in this review, all the proteins which form complexes with metals which are thermodynamically stable in a given chemical environment and kinetically stable on the time scale of the analytical procedure, are referred to as metalloproteins. The term: metalloproteome will refer to the collection of metal-site structures in all proteins of the proteome of a

given organism grown under a given set of conditions (or of a given tissue) in a given stage of development. Metalloproteins and their bound metals could consequently be considered as biological markers for physiological differences or pathological changes in human tissues. A comparison of the element binding pattern in cytosols of different human organs was then possible.[39]

The major groups of proteins of interest include metalloenzymes, metal-transport-proteins and metal-stress proteins. The analyst's task consists of the identification of the protein ligand, demonstration of the presence of the complex in the biological environment and the determination of the metal stoichiometry in it. An emerging trend is the development of high-throughput screens of protein-binding transition metal ions for use in assaying all of the proteins in a given proteome (*e.g.* yeast, serum) to identify the corresponding metallome.

Metalloenzymes. Metal ions are essential cofactors for functional expressions of many proteins in living organisms. In cells, several trace elements are needed to activate and stabilize enzymes, such as superoxide dismutase, metalloproteases, protein kinases, and transcriptional factors containing zinc finger proteins. The elements concerned are usually transition metal ions with small atomic radii (Cu, Fe, Zn, Mn, Mg, Ni) and interact *via* both electromagnetic as well as electrostatic attraction, making such coordinating interaction the strongest of the metal–protein interactions known. In proteins metals are present in the catalytic sites of redox proteins, metalloproteases and related metal-binding hydrolytic enzymes. The unique metal requirements of microorganisms are a target in the development of antibiotics for medical applications.

Metal-transport proteins. In the most popular understanding they are cysteine-rich proteins such as albumin or transferrin which assure the transport of heavy and essential elements in the human body. Recently, intracellular metal trafficking proteins, metallochaperones, that carry metal ions to specific target proteins, have been identified in various organisms.[40–42] An emerging field which can profit from the advances in hyphenated techniques is research on the mechanisms of metal sensing specificity. Proteins that regulate metal homeostasis, such as transcription factors, can differentiate between the appropriate metal and all the other metals that are present in the cellular environment, some at much higher concentrations. These studies aim at engineering organisms as biosensors or in a rapidly growing field of environmental bioremediation.

Metal-stress proteins. Some organisms defend themselves against a heavy metal induced stress by the synthesis of proteins able to complex the excess of the metal. The best known group of these is metallothioneins which are a group of non-enzymatic low molecular mass (6–7 kDa), cysteine-rich metal-binding proteins, resistant to thermocoagulation and acid precipitation.[43] They are considered to intervene in the metabolism, homeostatic control, and detoxification of a number of essential (Zn, Cu) and toxic (Cd, Hg, As) trace elements. The analytical chemistry of metal complexes with MTs was reviewed.[29,44]

3.1.4. Elemental tags for quantitative proteomics. Another possibility of detecting and quantifying proteins is tagging a particular protein (or a particular class of proteins) with a molecule containing an element to which ICP MS would be sensitive. The use of ICP MS in this context was pioneered by Baranov *et al.* who developed a very sensitive immunoassay using gold-tagged antibodies.[45] After reaction with the protein of interest the protein was quantified *via* the Au signal by ICP MS.

It should be noted that a number of elemental tags for proteins and peptides exist but have never been used in combination with ICP MS. Metal-clusters of *e.g.* tungsten, silver, platinum, gold or iridium were explored as tags for biological structures to make them visible in electron microscopy. Undecagold7 containing 11 gold atoms as well as Nanogold7 with *ca.* 67 gold atoms are meanwhile commercially available for labeling of proteins or peptides at the cysteine, histidine or *N*-terminal groups. Probes utilizing the fluorescent properties of Eu, Tb, Dy or Sm chelates are used to measure the concentrations of various antigens in an automated immunoassay system

Recently dinuclear zinc(II) complexes designed to bind specifically to phosphorylation sites in proteins were developed.[46] and applied to rapid and sensitive analysis of phosphorylated compounds ($ROPO_3^{2-}$) by MALDI-TOF MS.[47,48] The use of single Zn-isotope derivatives ($^{64}Zn_2L_3^+$ and $^{68}Zn_2L_3^+$) enables improvement of the sensitivity and accuracy of the analysis. Although Zn is not the best metal for the sensitive detection because of the risk of contamination this type of approach with other metals is likely to proliferate in the near future.

3.2. Small-size biomolecules with a covalently incorporated heteroelement

3.2.1. Organoselenium compounds. Selenium exists in biological systems in the form of inorganic species such as Se(IV) (SeO_3^{2-}-selenite), Se(VI) (SeO_4^{2-}-selenate) or selenides (*e.g.* HgSe), or in the form of organic species having a range of molecular masses and charges, starting from the simplest MeSeH and ending at complex selenoproteins.[49]

The essentiality of selenium results from its presence as a necessary component to form the active center, selenol group (–SeH), of glutathione peroxidase, thioredoxine reductase and of other selenoenzymes. Cancer chemopreventive effects were observed in the case of inorganic selenium salts, selenoamino acids and various synthetic organoselenium compounds. The metabolism of inorganic Se is complex and involves a number of species of which the most important are selenoamino acids: selenomethionine in microorganisms and plants, and selenocysteine in animals and man.

The recognition of the protective role of selenium supplementation against cancer[50] and the wider availability of selenized-yeast for this purpose raises concerns about the quality, safety and origin of marketed preparations.[51] These issues can be addressed by appropriate analytical methodology allowing the characterization of selenized yeast in terms of the identity and concentrations of selenocompounds present.[52,53] Selenium-accumulating plants provide unique tools to help us

understand selenium metabolism. They are also a source of genetic material that can be used to alter selenium metabolism and tolerance to help develop food crops with enhanced levels of cancer-protecting selenium compounds, as well as plants for the phytoremediation of selenium-contaminated soils.[54]

Selenium is excreted into urine. In addition to trimethylselenonium ions the major metabolites were recently identified to be selenosugars.[55,56]

The major fields of interest include: (*i*) speciation of metabolite products (*e.g.* amino acids, peptides, nucleosides) in microorganisms, plants, and nutritional supplements, and (ii) speciation of selenium metabolites in urine.

3.2.2. Organoarsenic compounds. The metabolism of inorganic arsenic by marine and terrestrial plants and animals leads to the formation of a range of organic arsenic species that may be considered as naturally occurring compounds.[57] The most widely referred to of this group is a quaternary arsenocompound: arsenobetaine, which is the major organoarsenic compound in marine animals, and arsinoyl-ribosides (arsenosugars) which are products of the As metabolism in marine plants and some bivalves. Besides compounds with a covalent As–C bond, As(III) may form complexes with thiol groups of glutathione (GSH) or proteins.[58,59] Another field of interest includes studies of the metabolism of arsenic following its administration to humans and experimental animals by means of the determination of arsenic speciation in urine. Chemical structures and names of organoarsenic compounds of interest for bioinorganic speciation studies were summarized in a recent review.[57]

3.2.3. Phospholipids. Phospholipids are complex lipids, which contain as their backbone glycerol esterified in the sn-3 position with a phosphate residue. The interest in phospholipids results from their being constituents of membranes in all types of cells in plants, animals and microorganisms. Phospholipids have attracted attention as biomarkers in chemotaxonomic studies and in producing liposomes for drug delivery or cosmetics.

Chemical structures of the most common phospholipids were summarized elesewhere.[60] The glycerol-3-phosphate is esterified at its sn-1 and sn-2 positions with fatty acids (typically 16–18 carbon atoms), and at its phosphoryl group to an alcohol, to form various phospholipids classes. The basic phospholipid structure is phosphatidic acid, or 1,2-diacylglycerol-3-phosphate.[60]

The complexity and heterogeneity of phospholipids are responsible for a number of difficulties in analytical characterization. The current methods such as normal phase HPLC with MS and/or evaporative light scattering detection suffer from the need for the compound-dependent calibration, and the resulting problems in quantitative analysis. Hence, the interest in species-independent detection of ^{31}P in HPLC by ICP MS.[27,60]

3.2.4. Drug metabolites. Drugs often contain a heteroelement such as a metal (*e.g.* Pt, Ru, Au) or a non-metal (Cl, Br, S or P) which can be detected by ICP MS. Stable metal complexes are of interest in pharmacology since they are used in many areas, such as cancer treatment (Pt and Ru drugs), arthritis treatment (Au), or contrasting agents (Ga). The usually covalent character of the metal–carbon bond in metallodrugs allows the preservation of the heteroelement-containing moiety when the drug undergoes degradation or another metabolic process. The presence of a heteroatom also allows the monitoring of interactions of a drug with proteins and other molecules present in body fluids. Till recently, the use of ICP MS had been limited to metallodrugs, such as *cis*-platin and related compounds.[1,61] Advances in technology, however, and in particular a wider availability of collision/reaction cells on ICP MS systems, have made possible a sensitive detection of non-metals such as chlorine, bromine, iodine, sulfur and phosphorus. Consequently, metabolites of a number of other drugs can be studied.[27,62,63]

The studies of metallodrugs fall into four major categories: (i) studies of the drug purity, stability and chemical transformations that occur under physiological conditions, (ii) studies of interactions of drugs and their metabolites with biologically relevant molecules (amino acids, proteins, nucleotides, DNA fragments), (iii) studies of the kinetics of metal binding with blood plasma, and (iv) studies of the metabolism of a drug upon incubation with blood plasma.

3.3. Metal complexes with non-proteinaceous molecules

3.3.1. Metal-binding metabolites. The mechanisms of resistance of many organisms to toxic elements include high turnover of organic acids such as *e.g.*, phytate, malate, citrate, oxalate, succinate, and induction and/or activation of antioxidant enzymes, such as *e.g.* superoxide dismutase or glutathione peroxidase.[64] A well known mechanism of enhancing heavy metal accumulation and tolerance in plants is the expression of metal-binding peptides.[65] Of particular interest are hyperaccumulating plants which have developed efficient metal homeostasis mechanisms that allow them to live and reproduce in metal-rich environments.[66–68] The complexation of metals leads to a number of relatively poorly characterized metal complexes. The understanding of mechanisms controlling the detoxification can benefit from the identification of the species formed.

Among the ligands potentially responsible for binding metals in plants an important role is played by hydroxy acids (*e.g.* citric or malic acid) and non-proteinaceous amino acids, referred to as metallophores.[69] The best known family of metallophores are the derivatives of mugineic acid.[69] Present in root exudates they help solubilize essential elements, such as, *e.g.* Fe(III) from soils but their role (especially of nicotianamine) as transporters of metals in hyperaccumulating plants is appearing.

Phytochelatins (PCs) are a class of oligopeptides composed only of three amino acids: cysteine (Cys), glutamic acid (Glu) and glycine (Gly) and in which glutamic acid is linked to cysteine through a γ-peptide linkage. Their general formula is $(GluCys)_nGly$ where n is comprised between 2 and 11.[70] PCs can detoxify these metals by forming a metal–PC complex in which the metal is bound to the thiol group of the cystein unit. The general structure of phytochelatins is conservative in a wide variety of plants but some modifications may occur on

the *C*-terminal amino acid leading to the loss of glycine or its replacement by β-alanine, serine, glutamine or glutamic acid.[70]

3.3.2. Polysaccharides. Metals are readily complexed by uronic acid derivatives which are components of cell walls.[71] Plants contain significant concentrations of polysaccharides of which the potentially negatively charged oxygen functions can bind cations electrostatically and even chelate them *via* polyhydroxy groups.[71] In comparison with proteins, however, little is known about the relevance of metal coordination to carbohydrates that are the most abundant (by weight) class of compounds in the biosphere. Attention was attracted by a structurally complex pectic polysaccharide rhamnogalacturonan-II (RG-II).[72,73] This ubiquitous component of primary plant cell walls forms dimers cross-linked by 1 : 2 borate diol esters (dRG-II) which were found to complex *in vitro* specific divalent cations[74] and the majority of Pb, Sr, Ba and REEs in fruit and vegetables.[75,76]

4. Advances in hyphenated systems using ICP MS detection

Research toward understanding the mechanisms of interactions of trace elements and metal probes with bioligands requires analytical techniques which are able to provide information on the identity and concentrations of element species occurring in biological tissues at the picogram and lower levels. The acquisition of this type of data is possible with hyphenated (coupled) techniques that combine a high resolution separation technique with sensitive element or molecule specific detection.[14,18,77] The current state of the art of these techniques for the bio-inorganic speciation analysis is schematically shown in Fig. 2. The increasing use of ICP MS for biochemical applications during the past 5 years is inseparably linked with the progress in three major areas including (i) advances in the detection of non-metals, (ii) increase in absolute detection limits of a new generation of ICP mass spectrometers allowing the coupling of capillary and planar electrophoretic techniques, and (iii) democratization of the access to stable metal isotopes, including isotopically labeled biomolecules.

4.1. Elemental detection with ICP MS

A general advantage of the ICP as an ion source is its capability to generate primarily monoatomic positive ions from most elements. Thus the elements present can be identified on the basis of their atomic mass spectra without the many isotope peaks that complicate the mass spectra of parent ions of biopolymers. The ICP is also a multielement ion source. Several elements can be identified in the same chromatographic or electrophoretic peak by scanning or hopping the mass analyzer. The advantage of ICP MS is the independence of the signal intensity of the molecular environment of the determined element and thus the possibility of using inorganic elemental standards for the quantification of proteins and other biomolecules.[78] Indeed, as long as the molecule concentration was sufficiently low, the matrix interference from the surrounding molecule was found to be almost negligible and inorganic quantification standards could be used for quantification with an accuracy of 10% or better.[78]

For most elements ICP MS is a routine detection technique in chromatography and was extensively discussed elsewhere.[77,79–81] However, some biologically important elements, such as *e.g.* sulfur and phosphorus, have high ionization energies and are not as efficiently ionized in the ICP as metals. Moreover, they suffer from a number of polyatomic interferences and, until recently, they could not be determined by ICP MS in biological matrices. The problem of the poor

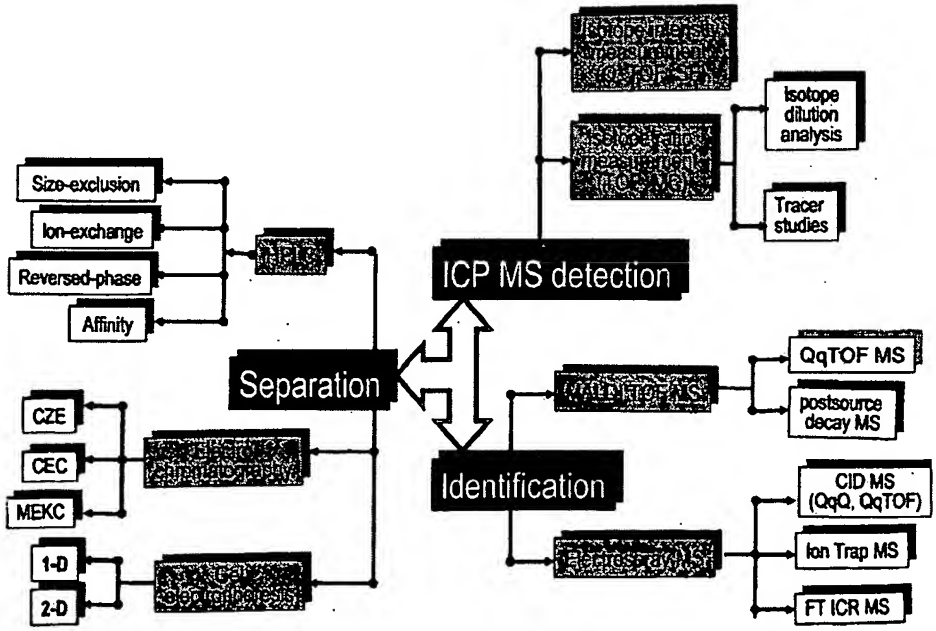

Fig. 2 Hyphenated systems using ICP MS detection.

ionization efficiency also concerns halogens, especially fluorine, which are of interest in drug metabolism studies. Also, some vital transition elements, such as *e.g.* iron or vanadium are interfered by polyatomic ions.

Polyatomic interferences can be removed by the use of sector field double focusing (high resolution) mass spectrometer or by using, in front of a quadrupole mass analyzer, a collision/reaction cell. The latter offers two possible approaches to the chemical resolution of interferences: (i) either by collision/reaction of the interfering polyatomic ions with gases such as H_2, He or Xe, or (ii) by reaction of the ion of interest with oxygen. In the first case a polyatomic ion is destroyed and thus removed from the *m/z* range of interest whereas in the second case a new product ion is formed and detected at a new, non-interfered *m/z* value.

In comparison with the quadrupole mass filters, sector instruments provide extremely low instrumental background, improved sensitivity in the low resolution mode for isotopes that are not prone to spectral interferences and very good detection limits (0.1–1 ng ml^{-1}) for nearly all elements. An increase in resolution, however, entails a loss of transmission and hence of sensitivity. A decrease in transmission also takes place when a collision cell is operating. Nevertheless, P and S are fairly abundant in biological samples and can be readily measured in the relatively low-transmission mode.

4.1.1. Detection of sulfur. Sulfur in biomolecules has been traditionally determined by ICP AES as reviewed for the HPLC-ICP AES quantification of metallothioneins.[29] However, the mass sensitivity was quite low limiting the success of the optical spectrometric detection to sulfur-rich proteins in standard (4.6 mm id) column chromatography.

In ICP MS the main isotope of sulfur ^{32}S (abundance 95.018%) suffers from a serious interference at nominal *m/z* 32 by the oxygen dimer ($^{16}O^{16}O^+$). Other interferences such as $^{14}N^{18}O^+$ (32.00223) and $^{15}N^{16}OH^+$ (32.00285) are minor but should nevertheless be considered. A resolution setting of 1801 is theoretically necessary to separate ^{32}S at *m/z* 31.97207 from its major interfering polyatomic ion $^{16}O^{16}O^+$ at *m/z* 31.98982.[82] However, in practice a higher resolution (at least 3000) is required, because the interfering species is much more abundant than $^{32}S^+$, and there is a distinct low-mass tail. The $^{32}S^+$ blank is made up of two components: a sulfur blank in the reagents and the tail from the $^{16}O^{16}O^+$ peak. It is possible to reduce the $^{16}O^{16}O^+$ tail by desolvating the mobile phase. However, the majority of the background is likely to be caused by the reagent and instrumental blank.

It was shown that, without solvent removal, the relatively poor abundance sensitivity of magnetic sector field instruments limits the background equivalent concentration to 10 ng mL^{-1} even at a resolution of 4500.[83] In similar conditions Evans *et al.* reported detection limits of 0.6–2 ng mL^{-1}.[82] The use of membrane desolvation allowed the reduction of these values by a factor of 100, down to 0.01 ng mL^{-1}.[84]

It should be noted that sulfur usually occurs in biological extracts at sufficiently high concentrations to allow the use of the minor ^{34}S isotope for quantification. In this way, the interference from the long front edge of the background peak from $^{16}O_2^+$ is prevented.

Recently quadrupole MS was shown to offer competitive figures of merit owing to the application of the collision/reaction cell technology. In the simplest case a quadrupole ICP MS tuned to generate high oxide formation rate can be used. Sulfur is measured interference-free as the oxide at *m/z* 48.[85,86] This concept was refined and the detection limits improved by carrying out the reaction with O_2 in a dynamic reaction cell (DRC).[87] A detection limit as low as 0.2 ng mL^{-1} of S was reported.[87] A similar approach was proposed using a hexapole collision cell.[88] The detection limit was 0.8 ng for sulfur, representing a 100-fold gain in comparison with the conventional detection mode.[88]

An alternative to the oxide formation is the collision induced dissociation of the interfering polyatomic ions. The $^{32}S^+$ and $^{34}S^+$ sulfur isotopes were detected using a He–H_2–Xe mixture in a hexapole collision cell with detection limits of 20–50 ng mL^{-1} for sulfur.[89] By using xenon as the collision gas the background was decreased by 6 orders of magnitude which allowed a decrease of the detection limits down to 1.3 ng mL^{-1} (^{34}S) and 3.2 ng mL^{-1} (^{32}S).[90] The presence of xenon reduced the ion transmission of all the measured elements, which resulted in the lower sensitivity in comparison with standard conditions.[90]

Detection limits in DRC MS and SF MS measurements were compared by Hann *et al.*[91] Whereas 4 ng of ^{32}S could be detected as the SO ion using the former, the detection limit was three times worse with the high resolution instrument.

4.1.2. Detection of phosphorus. Quantitative determination of phosphorus in biological samples can yield important information about the state of phosphorylation of proteins. The currently used method of phosphorus detection (γ-^{32}P labeling with scintillation counting) has an obvious drawback of the necessity of radioisotope handling and disposal. ICP MS is a convenient alternative offering the same response for ^{31}P regardless of structure of the molecule in which it is incorporated. However, phosphorus has a high ionization potential and is poorly ionized in the plasma.

The main polyatomic interferences at *m/z* = 31 are $^{15}N^{16}O^+$, $^{14}N^{16}O^1H^+$, and $^{12}C^1H_3{}^{16}O^+$, with approximate partition of 15 : 8 : 1. They are responsible for the combined $^{31}P^+$ background equivalent concentration of 10 μg mL^{-1} for a 3% acetonitrile sample matrix.[92] These values are instrument dependent but give a general idea of the difficulties encountered. The separation of these polyatomic interferences requires a mass resolution higher than 1500.[93] The phosphorus determination may also be disturbed with the double-charged atomic ion at $^{62}Ni_2^+$.

Similarly as for sulfur, the polyatomic isobaric interferences at *m/z* 31 can be avoided by the detection of a product oxide ion PO^+ formed in a DRC pressurized with oxygen.[87] A detection limit of 0.06 ng mL^{-1} P was reported. However, new interferences at mass *m/z* 47 (*e.g.* $^{47}Ti^+$, $^{15}N^{16}O_2^+$) need to be considered. A detection limit of 1–2 pg P was reported by monitoring ^{31}P directly in capillary electrophoresis using a DRC equipped mass spectrometer.[94] When a hexapole collision cell was used, the detection limit was 1 ng P which represented a 50-fold gain in comparison with the conventional mode.[88]

The detection of phosphorus can benefit from the reduction of the solvent load in the plasma to reduce the abundance of $^{15}N^{16}O^+$ and $^{14}N^{16}O^1H^+$ ions. A membrane desolvation system was reported to allow the phosphorus detection at the sub-μg mL^{-1} levels (with H_2 pressurized hexapole cell) but some compounds were lost during desolvation.[92] In another work, helium was used as collision gas.[60] Solvent load to the plasma was reduced by splitting the mobile phase prior to reaching the nebulizer, by chilling the spray chamber to −5 °C and by optimization of carrier gas flow for maximum condensation of organic vapours.[60] The detection limits obtained for phospholipids were 0.2–1.2 ng.[60]

It is worth reminding that phosphorus is an abundant element in the earth's crust which results in a risk of contamination of protein samples with free phosphates originating both from ambient fluids or from the laboratory environment, including chemicals.

4.1.3. Detection of other elements. ICP collision cell MS was comprehensively optimized for the simultaneous determination of P, S, Cl, Br and I-containing pesticides.[95] The reaction/collision cell technology allowed a considerable improvement of the selenium detection in chromatography of biological extracts. The removal of the Ar_2^+ interference allowed the use of the most abundant ^{80}Se isotope for quantification and thus a 5–10 fold decrease of detection limits.[96] The elimination of the interferences with the other isotopes enabled a wider use of isotope dilution quantification.[97]

As far as transition metals are concerned, ^{51}V is interfered with polyatomic ions such as $^{35}Cl^{16}O^+$, $^{38}Ar^{13}C^+$ and $^{37}Cl^{14}N^+$ whereas ^{56}Fe is interfered with $^{40}Ar^{16}C^+$ and $^{40}Ca^{16}O^+$.[98,99] The interfering polyatomic ions of $^{35}Cl^{16}O^+$, $^{40}Ar^{12}C^+$ and $^{40}Ar^{16}O^+$ on $^{51}V^+$, $^{52}Cr^+$ and $^{56}Fe^+$, respectively, were reduced significantly by using NH_3 as the reaction cell gas in the DRC.[100]

An interesting feature of ICP MS is the detection of carbon to provide a universal method for detecting organic compounds in chromatographic effluents. Another advantage is the possibility of the use of the carbon-heteroelement ratios as a means of internal standardization. Carbon is a difficult element to ionize and its detection suffers from a high background. $^{12}C/^{13}C$ isotope ratios were measured by ICP MS in aqueous solutions of tryptophan, myoglobin, and β-cyclodextrin using C^+ ions offering a precision of 1%.[101] The use of a narrow bore column with highly isotopically enriched ^{12}C-methanol (99.95%) as organic modifier for the mobile phase enabled the detection of *ca.* 0.1 mM of ^{13}C-triple-labelled caffeine and ^{13}C-double-labelled phenacetin.[102]

The monitoring of ^{14}C by its mass instead of its radioactive decay offers a new way to gain insight into the preservation of integrity of the organic part of drugs after administration. In a study of a ^{14}C labelled organoplatinum drug, Pt and ^{14}C were simultaneously monitored by ICP-MS.[103]

4.2. Chromatography with ICP MS detection

The coupling of HPLC employing different separation mechanisms with ICP MS has been the most widely used. Gas chromatography in bioinorganic speciation analysis has a limited area of application but can be of choice in some cases, such as, *e.g.* analysis of sulfur- and selenium-containing amino acids (after derivatization),[104–107] or of volatile sulfur and selenium[53,108] species. Accurate determination of selenoamino acids can be achieved by isotope dilution GC-MS.[105,106,109] The potential of GC methods for the chiral separations was demonstrated for selenomethionine enantiomers.[110–112]

The applications of HPLC-ICP MS were reviewed.[80] In particular, the potential of capillary and nanoflow HPLC-ICP MS coupling was discussed.[113]

4.2.1. Introduction of organics-rich HPLC mobile phases into the ICP. Reversed-phase HPLC is the preferred separation technique for weakly polar compounds in application areas such as peptide mapping, and amino acid, phospholipid and protein separations. The chromatographic analysis often requires the use of mobile phases rich in organic solvents, up to 100% of methanol or acetonitrile. The introduction of an organic solvent above a certain concentration level (usually 20–30% methanol and 10% acetonitrile at 1 mL min^{-1}) into the ICP MS is known to affect negatively the ICP stability and to lead to a decrease in signal intensity and to the deposition of carbon on the cones. The removal of the solvent vapor using a cooled spray chamber or a membrane desolvator accompanied by the addition of oxygen to the plasma gas and, consequently, the use of platinum cones, are the usual measures taken.

The influence of an organic modifier in the analyte solvent on the detection sensitivity was tested for sulfur, phosphorus and iodine.[114] Phosphorus and iodine show an increased signal proportional to the concentration of organic modifier with an intensity maximum at 40–60% which corresponds to metal-like characteristics. For the detection of sulfur this characteristic is counteracted by a signal suppression effect proportional to the amount of organic modifier.[114]

Trends in HPLC using organics-rich mobile phases include a post-column eluent splitting or dilution and/or the reduction of the flow rate. In an application concerning the phospholipid analysis solvent load to the plasma was reduced by 5-fold splitting the mobile phase prior to reaching the nebulizer, by chilling the spray chamber to −5 °C and by optimization of carrier gas flow for maximum condensation of organic vapours.[60] In a study of the levothyroxine degradation products the mobile phase containing above 20% acetonitrile at 300 μl min^{-1} was diluted (1 : 3.3) post-column on-line with a 2% (v/v) nitric acid solution.[115]

The use of capillary HPLC (flow rate of 4 μl min^{-1}) and nano HPLC (flow rate of 200 nL min^{-1}) is likely to alleviate problems with organics-rich mobile phases. At such low flow rates introduction of up to 100% of organic solvent becomes possible without either cooling the spray chamber or oxygen addition.[116] A considerable loss of signal intensity was nevertheless observed at acetonitrile concentrations exceeding 80% introduced into and ICP at 4 μL min^{-1}.[116]

4.2.2 Capillary and nano HPLC-ICP MS. The need for analysis of microsamples, such as, for example, digests of spots in 2D electrophoresis, compartments of individual cells, or human biopsy extracts, has spurred the development of nanoflow separation techniques in the past decade. Despite

the increasing popularity of electrochromatographic techniques, such as capillary electrophoresis and capillary electrochromatography, the position of capillary HPLC is still dominant.

Indeed, HPLC is a robust, reliable, and reproducible separation technique for various types of samples. It provides high resolution, especially when used in gradient mode, and can be easily implemented to various sample types and application areas. It can be scaled down to nanoflow dimensions and used in single or multidimensional separations. However, the capillary HPLC techniques are *a priori* incompatible with ICP MS because of the flow rates being 100–1000 times lower than those (0.7–1 mL min^{-1}) required by conventional nebulizers. Also, the large dead volume (40–100 cm^3) of the double-pass Scott spray chamber results in long washout times and peak broadening. Recently, however, a number of dedicated interfaces between capillary HPLC and ICP MS were reported.

Three different micronebulizers were tested: the MCN 6000,[92] the PFA 100,[26,37,92,117] and the DIHEN.[114] The disadvantages of the former two included the sample uptake rates of 10–100 μL, which were still higher than those demanded by capillary HPLC (4 μL), and the large dead volume of the desolvation system of the MCN 6000. Even with the modified DIHEN, the peaks were relatively broad (15 s peak width at half-height).

A direct injection high efficiency nebulizer (DIHEN) was modified for μLC-ICP-MS by inserting an additional internal capillary with 90 μm od and 20 μm id to minimize the interface dead volume. The modified DIHEN was compared with a conventional microflow nebulizer with a spray chamber interface in terms of sensitivity, signal stability and organic modifier dependency at flow rates ranging from 0.5 to 5 μL min^{-1}.[114] Although the modified DIHEN was slightly less sensitive, its signal stability was found to be superior, and its chromatographic resolution was much better due to its extremely small dead volume. These characteristics were demonstrated during the analysis of a mixture of synthetic phosphopeptides (^{31}P detection) and synthetic thyroxine (^{127}I detection).[114]

A sheathless interface based on a total consumption micronebulizer operating at flow rates in the range 0.5–7.5 μL min^{-1} was developed between capillary HPLC and ICPMS by Schaumlöffel *et al.*[116] It allowed the efficient nebulization and transport into the plasma of mobile phases containing up to 100% organic solvent without either cooling the spray chamber or oxygen addition. The minimal peak broadening (5 s at half height) allowed baseline resolution of a mixture containing more than 30 selenopeptides, many of which could not be separated using the conventional HPLC-ICPMS coupling.[116]

A capillary HPLC-ICPMS coupling[26,37,92,117–119] was optimized for ^{31}P detection and used for the analysis of phosphopeptides[114] in a tryptic digest of β-casein[92] and the identification of the phosphorylation sites in phosphoproteins.[37,118]

The coupling of nanoflow HPLC to ICP MS using a sheath flow allowing non-specific isotope dilution quantification was developed by Giusti *et al.*[120] The peak width at half-height was reduced to 2.3 s for a 11 nL sample volume injection. It is evident that such small injection volumes are highly impractical in view of ICP MS being sensitive to the mass of an analyte and not to its concentration. Therefore, these approaches should be combined with a preconcentration step which can be easily automated using commercial 2D HPLC systems.

Fig. 3 compares chromatograms obtained using normal bore, capillary and nanoflow chromatography for the mapping of selenopeptides in a protein fraction isolated from a selenized yeast extract.[116,120] The decrease in the column dimensions improves the separation efficiency and reduces the absolute detection limits from *ca.* 10 pg with a 4.6 mm column to 150 fg in cHPLC-ICP MS to *ca.* 50 fg in nanoflow HPLC-ICP MS.

4.3. Capillary electrophoresis-ICP MS

Fundamentals and the practical aspects of interfacing CE with ICP MS were reviewed.[81] The overwhelming majority of successful applications were based on the interface developed by Schaumlöffel and Prange[121] and commercialized by CETAC (Omaha, NE) although some other elegant interface designs were reported.[122,123] The commercial interface successfully solves three basic problems occurring when coupling CE with ICP MS. They are: (*i*) the efficient introduction of the nanoflow effluent to the ICP MS realized by the dedicated total-consumption micronebulizer (5 μL min^{-1}) and a low-dead volume spray chamber, (*ii*) the closing of the electrical

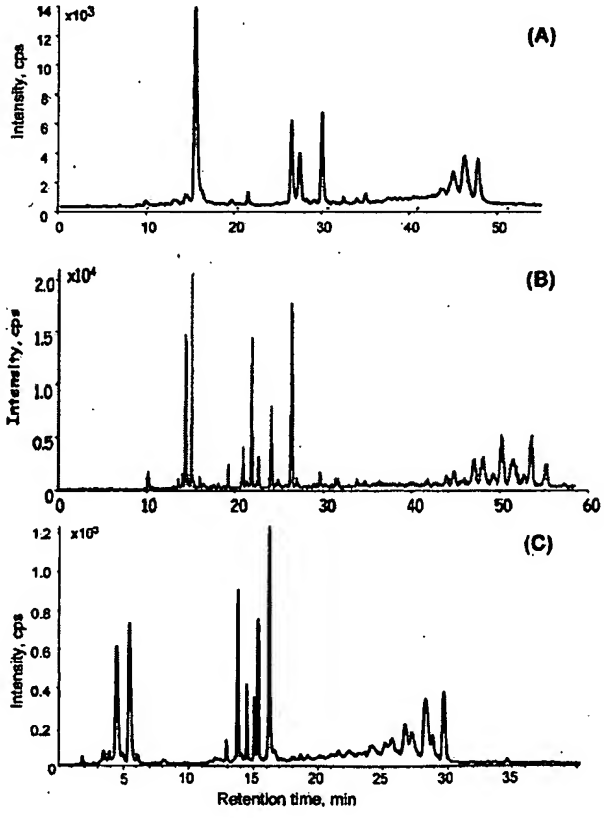

Fig. 3 Comparison of chromatograms obtained using (A) normal bore,[116] (B) capillary[116] and (C) nanoflow[120] chromatography for the mapping of selenopeptides in a protein fraction isolated from a selenized yeast extract.

circuit in the interface by inserting an electrode into a sheathflow, (*iii*) the prevention of the laminar flow by the optimization of the fluid dynamics.

Most applications reported were carried out using a sector field high resolution mass spectrometer. This is likely to be due to the fact that the target analytes included sulfur determined along with the metals. The coupling of CE to ICP TOF MS for multielemental speciation analysis was described but no applications to the analysis of metallobiomolecules were reported.[124] The use of collision cell quadrupole MS detection of phosphorus in CE was reported with brighter perspectives for a wider implementation than ICP TOF MS.[94]

The feasibility of the CE-ICP MS coupling in bioinorganic speciation analysis was often demonstrated for a mixture of pure standards of heteroatom-containing biomolecules such as, *e.g.* monophosphate nucleotides,[94] cobalamine and its analogues,[125] and Co, Mn, and Zn protoporphyrins.[126] The rapidly developing application areas, discussed below, include the determination of stoichiometry of metal–protein complexes, metal-binding studies with pure compounds of biological origin, and the use of CE for fine (second or third dimension) separations of metal-containing fractions isolated by chromatography. The salt content of the sample is a principal limitation requiring an optimization of the sample desalting process prior to CE analysis.

A CE-ICP MS setup requires a rather bulky hardware but, recently, the use of microfluidic chips interfaced with ICP MS was proposed to provide rapid elemental speciation capabilities.[127]

4.4. Gel electrophoresis laser ablation ICP MS

Polyacrylamide gel electrophoresis, employed either in the monodimensional (isoelectric focusing or SDS) or 2D mode (IEF × SDS), is a routine high resolution technique for the separation of proteins.[128] Proteins containing a covalently incorporated heteroelement can be separated using the existing proteomics protocols (*cf.* the works of Behne *et al.*[31]) The separation of the non-covalent species, *e.g.* metal complexes with proteins requires non-denaturing conditions. The presence of SDS or the high voltage field are likely to lead to a loss of metal.

The classical detection technique remains the autoradiography of (^{75}Se,[129,130] ^{35}S, ^{31}P) with its inherently inconvenient need for radioactive isotopes. There is an increasing number of reports on the use of synchrotron radiation XRF for metalloproteins.[24,131,132] However, the largest interest is attracted by scanning of gels for the presence of heteroatom-containing proteins by laser ablation ICP MS which offers a cheaper and apparently competitive alternative.

This technique, pioneered by Nielsen *et al.*,[133] consists of the ablation of the analyte with a laser beam guided over the gel within an electrophoretic lane. The ablated analytes are swept into the ICP by a continuous stream of argon, and the ions are analyzed by MS. As a result, an electropherogram is obtained in which the quantity of a given element is a function of its position in the gel. Two ablation strategies: (i) ablation with translation on a lane relevant for 1D separations, and (ii) single-hole drilling relevant for ablation of spots after 2D separations, were developed.[96] Spotting and quantification by LA ICP MS is potentially a fast and a fairly robust technology, since no further reaction or derivatization step is involved and the signal is theoretically directly proportional to the quantity of the analyte element in the gel. It eliminates the problems related to the recovery of the protein from the gel for quantification and offers advantages over quantification methods based on cHPLC-ICP MS requiring the full sequence coverage.

The ablation can be carried out directly in the gel or from the membrane obtained after Western blotting.[119] The latter eliminates the problems due to the loss of the geometrical integrity of the gel in the aggressive drying process in the ablation chamber and allows the preconcentration of the analyte diffused through the gel on the surface of the blot. In the case of a gel, a danger exists that only the upper layer of the gel is ablated which results in worse detection limits. By the blotting process the proteins are enriched at the surface in a layer which can be ablated completely by scanning the laser across the electrophoretic lane.[119] Speciation of protein-bound trace elements by gel electrophoresis and atomic spectrometry was recently reviewed.[134]

To date applications focused on three areas: spotting of selenium-containing proteins,[96,135] spotting and determination of phosphoproteins,[119,136,137] and detection of metal complexes, usually with protein ligands.[137,138] LA ICP MS can enable the identification of drug-binding proteins, especially if they contain elements uncommon in biological systems.[139] The technique is particularly attractive for detecting metal–protein complexes formed in response to metal stress because of their usually high abundance.[138] Matching the levels of the endogenous metal-complexes in human tissues is still a challenge.[131,136] An example of the use of detection of metal-binding proteins in brain tissue extracts by 2D GE-LA ICP MS is shown in Fig. 4.

4.4.1. Contamination and background problems. Laser ablation ICP MS eliminates the needs for excision and digestion of protein spots from gel which are important sources of contamination. Nevertheless, the detection of P and S is limited by relatively high background concentrations around the spots. The impurities in the staining compounds might stick to the proteins more intensely than to the gel.[137] Problems with the high background are more intense when a gel is ablated directly. Indeed, contaminations can be removed more easily from the blot membrane than from the gel because of the higher chemical stability of the former. Also, the analyte is usually more concentrated in a blot than in a gel.

In the case of sulfur the high background is due to the use of sulfur-containing chemicals, *e.g.* ammonium persulfate used in polymerization of acrylamide and to sodium dodecylsulfate (SDS) used for protein denaturing.[139,140] In the case of phosphorus the background can be due to one of many buffers used in the electrophoresis process; modifying the staining and destaining procedures could provide some improvements.[141] A dedicated washing step was found to be necessary to remove phosphate non-covalently bound to proteins.[119] The contamination problem is especially valid for samples with

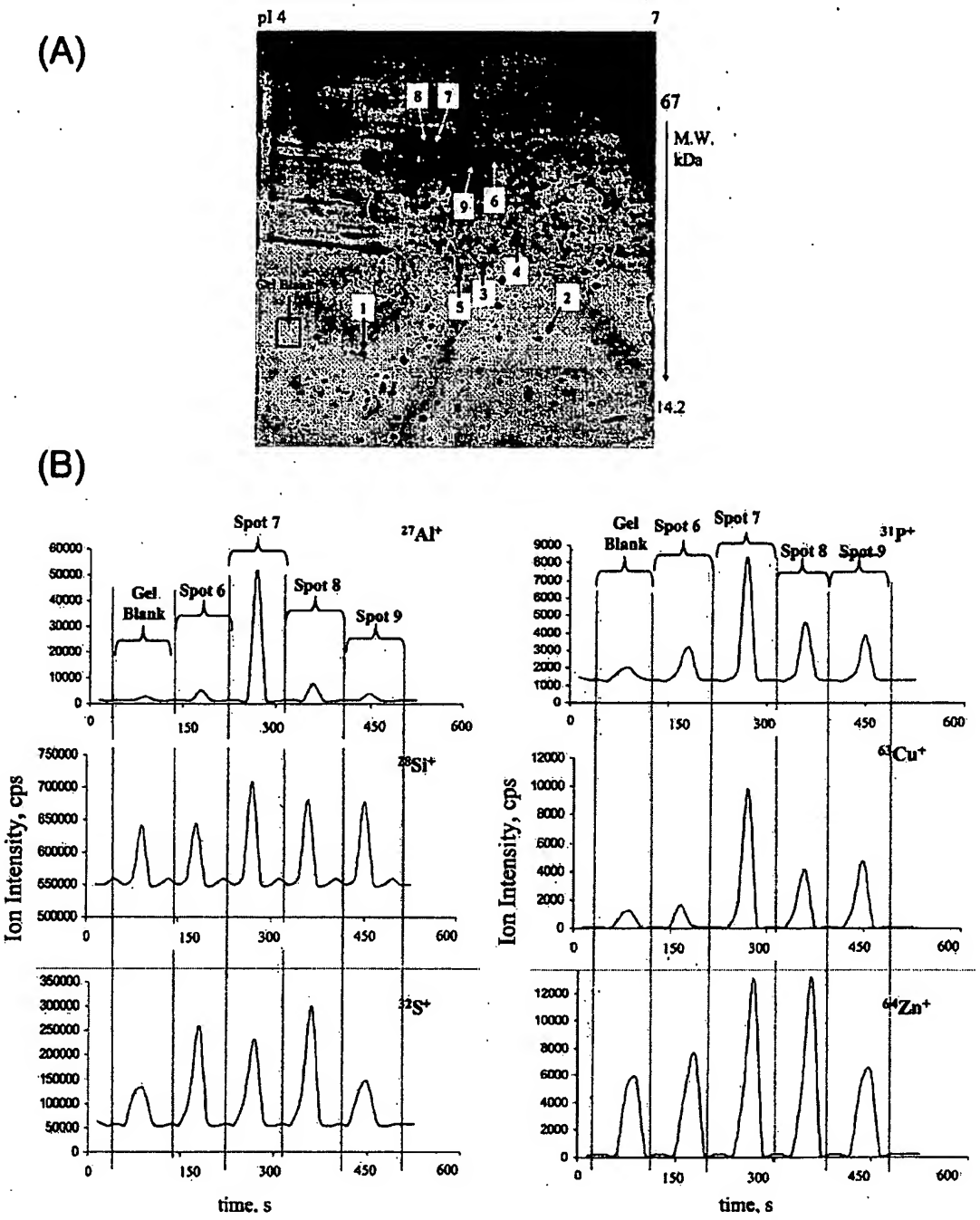

Fig. 4 Laser-ablation ICP MS detection of metals in protein GE spots.[136]

high initial P concentration such as serum or cerebrospinal fluid in which free phosphate is present as HPO_4^{2-} at millimolar concentrations.[137] The replacement of the Coomassie Blue staining by silver staining was reported to reduce the background by one order of magnitude for phosphorus and a factor of 7.5 for sulfur.[136]

4.4.2. Quantification, calibration and detection limits. The applicability of LA-ICP MS to the detection of trace elements in gels was systematically assessed for 16 elements and detection limits in two modes: ablation with translation and single-hole drilling, were tabularized.[96] Marshall *et al.* first discussed the spotting of a phosphorylated protein (β-casein) on 1D electrophoresis gel blots reaching a detection limit of 0.5 ng phosphorus (S/N 10 : 1) using a hexapole collision cell quadrupole ICP MS.[141] The detection limit found be Wind *et al.* using a high resolution ICP MS instrument was lower, 0.15 ng phosphorus.[119] Using ICP SF MS Becker *et al.*[136] reported for phosphorus a detection limit of 20 pg g^{-1} in comparison with a value of 1.3 ng g^{-1} obtained using a collision cell quadrupole instrument. The detection limits for sulfur were much higher 0.6 μg g^{-1}.[136]

For quantification the signal can be normalized to the ^{13}C response[139] or to that of an internal standard, *e.g.* Co, added to the gel at a defined concentration.[137] A solution based calibration strategy was proposed using an ultrasonic nebulizer for introduction of calibration standard solutions coupled to the laser ablation chamber.[136,137] These methods do not take into account the inhomogeneous in-depth distribution of the proteins.[136] Therefore the use of an internal standard is a better choice. Sulfur present in most of proteins is a natural choice provided that the target protein is known and contains either methionine or cysteine.[137] The simultaneous detection of S and P allows, in the case of homologous proteins containing cysteine and methionine the use of S as an internal standard for P quantification. Becker *et al.* showed that concentrations of P and S were changing with changing gel depth but the P/S ratio remained constant.[136,137]

Multielement scanning of the gels by LA-HR ICP MS is far from routine. The need for a relatively wide mass window to be scanned resulted in a longer scan time per isotope and was disadvantageous if multielemental species analysis should be performed.[136] The use of a multiple ion collector for this purpose would be beneficial.

5. Isotope dilution quantification in bioinorganic speciation analysis

The complexity of the biological sample and the multistep character of many bioinorganic speciation analytical procedures stimulate interest in isotope dilution analysis (IDA) to improve precision and accuracy of the quantification of biomolecules and the related metal complexes. The two principal approaches include speciated IDA (species-specific spike is used) and non-speciated IDA (when the isotopic spike ignores the speciation of the analyte compounds).[142]

In the speciated IDA an isotopically labeled analyte species is added to the sample and is supposed to co-elute from a chromatographic column with the analyte species after an entire analytical procedure. Since the basis of quantification is the measurement of the isotope ratio of the speciated element in the mixture producing a chromatographic peak, incomplete recoveries and matrix effects can be corrected for. The use of this approach is limited by the availability of a labeled analyte molecule and the equilibration of the spike with the analyte species. In the here reviewed area the principal application was the determination of selenomethionine in yeast and gluten,[105,106,109] and in serum.[97]

A method based on the species-specific IDA was developed for the accurate determination of an Asp-Tyr-SeMet-Gly-Ala-Ala-Lys peptide in a tryptic digest of an aqueous extract of selenized yeast.[143] For this purpose a ^{77}Se labeled peptide to be used as a standard had been purified from yeast grown on $^{77}SeO_4$-rich culture by 2D LC and quantified by reversed IDA. The sample mixed with the ^{77}Se labeled peptide spike was analyzed by capillary HPLC-ICP collision cell MS. The use of a labeled peptide allowed the correction for retention time shift and possible peak distortion due to the injection of a complex salt-rich matrix onto a capillary column. The isotope ratio of selenium ($^{78}Se/^{80}Se$) was measured in the peak corresponding to the peptide of interest allowing its accurate quantification. The determined concentration of the peptide, which was quantitatively formed from a selenized 12 kDa heat-shock protein, allowed the quantification of the latter by cHPLC-ICP MS directly in a yeast extract, without any additional purification.[143]

For most of biomolecules isotopically labeled calibration standards are unavailable and the only possibility allowing the improvement of precision and accuracy is the continuous introduction of an isotopically enriched, species-unspecific spike solution after the separation step. Quantification by external calibration gives rise to problems as a result of matrix-induced differences in detector sensitivity between standard and sample. The separation of possible MT isoforms in eel liver cytosol was carried out by SEC-AE-FPLC. Once the final separation is accomplished, a mixed solution containing the enriched isotopes ^{111}Cd, ^{65}Cu, and ^{67}Zn was mixed with the AE effluent and the ratios $^{114}Cd/^{111}Cd$, $^{63}Cu/^{65}Cu$, and $^{64}Zn/^{67}Zn$ were quantified on-line using an ICP-(Q)MS.[144] An on-line isotope dilution method using post-column addition of the enriched isotopes ^{65}Cu, ^{67}Zn and ^{106}Cd was developed for the quantification of metal-MT complexes by HPLC-ICP TOF MS.[145] IDA by ICP-MS was proposed for the determination of oxidized MT by a Cd-saturation method in rat liver cytosol[146] and selenium species in yeast and flour[147] and blood plasma[148] samples.

Species-unspecific IDA was used for the determination of the metal stoichiometry and quantification of MT complexes by capillary electrophoresis-ICP MS.[149,150] Based on the known number of S atoms (21) per metallothionein molecule each MT isoform could be quantified independently. Additional measurements of the metals Cu, Zn, and Cd, followed by the determination of the sulfur-to-metal ratio in MT, enabled the determination of the stoichiometry of a complex. A typical molar flow electropherogram allowing a direct reading of the metal stoichiometry is shown in Fig. 5.

Nanoflow HPLC-ICP MS IDA was developed for the quantification of selenopeptides in a tryptic digest of a selenium-containing protein.[120] ^{76}Se was introduced in the makeup flow.

6. Probing of protein–metal ion interactions, stoichiometries and relative affinities

To date, only very few methods allow the direct determination of the exact stoichiometry of the metal–protein binding which

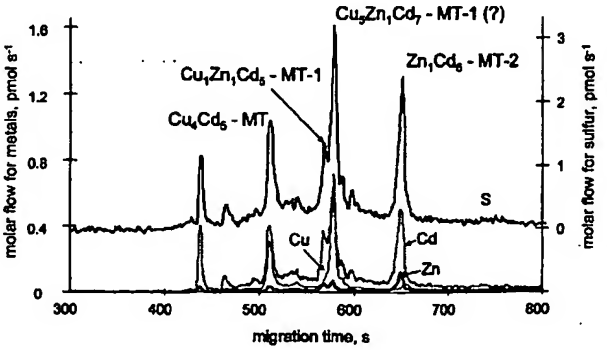

Fig. 5 A typical molar flow electropherogram allowing a direct reading of the metal stoichiometry.[150]

is necessary to get an insight into the mechanisms of protein–metal ion interactions. These issues are of particular concern for the quality control of metal integration in biotechnologically produced (heterologously expressed) metalloproteins. Conventionally, the molecular metal content of unknown or biotechnologically produced metalloproteins is assessed by determination of the total metal concentration in solution of isolated protein. The accuracy is compromised, especially by the potential presence of impurities as a result of the protein isolation procedure. Still a bigger challenge is the determination of the stoichiometry of metal complexes at the trace levels directly in biological cytosols. There is also a need for techniques which would allow investigations of reactions running at low- or even sub-μg L^{-1} concentrations of the substrates. The use of HPLC or CE with ICP MS or ES MS detection or the use of ES MS as a standalone technique opens a number of attractive possibilities in these areas.

6.1. ICP MS related techniques

6.1.1. Chromatography with ICP MS detection. The sulfur concentration value can be an accurate measure of the molar protein concentration, if the primary structure of a protein, and thus the number of sulfur containing amino acids, are known. The simultaneous determination of the metal and sulfur permits the metal/sulfur ratio, and thus the metal binding stoichiometry, to be determined.

In the case of a relatively pure sample the resolution of SEC may be sufficient to accomplish the separation of the metalloprotein from potential low molecular weight impurities prior to ICP MS detection. SEC-ICP-DRCMS was used for the determination of Fe/S and Mn/S ratios in five commercially available metalloproteins (myoglobin, haemoglobin, cytochrom c, arginase and Mn superoxide dismutase from *E. coli*). Two proteins were characterized after heterologous expression in a host organism (Mn superoxide dismutase from *Anabaena* and catalase-peroxidase from *Synechocystis*).[91] A similar technique confirmed that both iron (0.80 ± 0.09 mol subunit^{-1}) and zinc (0.43 ± 0.03 mol subunit^{-1}) were bound to the enzyme human endothelial NO synthase.[151] Another example concerned the measurement of the Gd/S ratio in metal-tagged contrast agents.[152]

When the resolution of SEC is insufficient, another separation mechanism may be of choice. Cation-exchange HPLC-ICP MS was developed for the determination of the Pt/S stoichiometry in adducts formed by *cis*-platin, monoaqua-*cis*-platin and diaqua-*cis*-platin with methionine.[153] Reversed-phase HPLC was employed to determine quantitatively the concentration of nucleotides from modified DNA in standard solutions based on the signal of phosphorus. Four molecular forms of transferrin: monoferric transferrin bound to the C-site, holotransferrin, apotransferrin and monoferric transferrin bound to the N-site were separated by HPLC using a pyridinium polymer column.[154] Anion-exchange HPLC-HR ICP MS was used to study the metal–transferrin binding patterns by the monitoring of ^{51}V, ^{56}Fe and $^{32}S^{98,99}$ or of ^{27}Al, ^{56}Fe and ^{32}S.[155,156] The affinity of Al and Fe to transferrin was compared between native- and asialo-transferrins, in order to clarify whether the presence of sialic acids influenced the metal binding.[156]

An innovative methodology based on ion-exchange HPLC-ICP MS was developed to study the ability of metallothionein to donate essential and non-essential metals to apo-carbonic anhydrase.[28] Stable isotopically labeled $^{67}Zn_3Cd_4$-MT-II was used to differentiate between the Zn donated from MT-II and that from extraneous sources.[28]

6.1.2. Capillary electrophoresis-ICP MS. Capillary electrophoresis is a well suited technique for the separation of metalloprotein and often its isoforms from metal ions and other species. Most CE-ICP MS applications concerned studies of binding of Cu, Zn and Cd, bound to either native or recombinant metallothioneins.[90,149,150,157] A number of mixed metal complexes with different migration times were observed during titration as a function of the Cu : Cd ratio.[158] Capillary electrophoresis is less prone (than HPLC) to artefacts due to the metal exchange by interaction with the stationary phase. The use of uncoated fused silica capillaries was privileged because of the simplicity, low cost and capillary lifetime.[113] On the other hand, coated capillaries were recommended to reduce the adsorption problems and to improve the separation efficiency.[159] Nine (in comparison with 3 in an uncoated capillary) MT complexes in a rabbit liver preparation were successfully separated on an anionic polymer coated column, prepared by immobilizing poly(2-acryloamido-2-methyl-1-propanesulfonic acid) on the fused-silica surface *via* a linking agent.[159]

The analysis of proteins with higher molecular mass increases the risk of adsorption of a protein on the capillary wall. Another problem is a lower contribution of the metal which makes the detection more difficult. CE-ICP-MS was proposed for a rapid assay for high-throughput study of *cis*-platin–human serum albumin interactions.[160] CE was used to separate free metal ions from the metal bound to metalloprotein, the latter being the only biologically relevant metal species. Reports to date concerned the determination of the stoichiometric ratio Zn/S in Zn-β-lactamase[161,162] and in carbonic anhydrase,[163] and the Cu : Zn : S ratios in Cu/Zn superoxide dismutase.[163]

A separate group of studies focused on interactions of metallodrugs with model molecules. The reaction kinetics of the *cis*-platin with 5′-guanosine monophosphate (5′-GMP) was followed *in-vitro* by monitoring the decrease in the concentration of 5′-GMP and the increase in the concentration of formed adducts by HPLC-ICP-SFMS.[164] The method provided stoichiometric information about the major GMP-adduct by the determination of the P/Pt ratio.[164]

6.2. Electrospray MS techniques

Electrospray MS has found widespread use in the determination of noncovalent interactions and functional proteomic analyses. Complexes of biological molecules, such as amino acids, peptides, proteins, and carbohydrates with transition metals are readily transferred to the gas-phase by electrospray ionization and their gas-phase and solution properties can be correlated.[165] The technique is highly specific and relatively

sensitive. A serious inconvenience with the use of electrospray for probing the metal–ligand interactions and the determination of metal–ligand stoichiometry is its poor tolerance to nonvolatile salt buffers and other solubilizing agents (*e.g.* detergents) which are often necessary to maintain the analyte's stability and integrity. An electrospray compatible solvent system containing a volatile salt such as ammonium acetate, ammonium formate or ammonium carbonate is required. The desalting and buffer exchange can be carried out off-line (infusion experiments) or on-line (by SEC or CE).

Size-exclusion chromatography can be conveniently used for on-line desalting and buffer exchange. Columns of 50 mm × 800 μm were found to be sufficient for this purpose.[166] With longer columns, the SEC approach could be effectively used both to separate proteins in a complex mixture and to exchange buffers prior to electrospray.[167] SEC-ESI MS was applied to the determination of Ca^{2+} binding stoichiometry to a high affinity metal binding protein calbindin.[167] The same approach can also be used to determine metal ion binding stoichiometries of low-affinity metal-binding proteins. With the latter, however, it may be necessary to add metal ions to the mobile phase in order to determine the stoichiometry of the protein–metal ion complex.[167] This was demonstrated for a study of the metal complexation by a protein involved in the induction of sporulation in a number of *Bacillus* species). Addition of metal ions to the mobile phase was reported to help overcome the metal stripping process by the stationary phase.[168]

A less convenient (because of the more complex hardware necessary) but equally efficient method for buffer exchange is capillary electrophoresis. It was reported to offer the possibility of the removal of salt from solutions containing up to 100 mM Tris or phosphate that would otherwise suppress the electrospray ionization.[169] The method was applied to studies of the metal displacement from the complexes with recombinant MT.[169,170]

Applications of electrospray MS to metal speciation studies in biochemistry were reviewed,[171,172] with particular emphasis on metal complexation by phytochelatins and metallothioneins.[173] In a careful study Zn complexes formed between MT and a series of peptides related to glutathione were examined by nanoES-QTOF MS.[174] Differences in the distribution of Zn ions between the pairs of dissociation products provided qualitative information about the biomolecular chelation of metal ions.[174] Other applications of direct ES MS included study of metal binding to brain-specific MT-3[175] and covalent iodine binding to fulvic acids.[176] Strategies for the sequencing of argentinated peptides by ES MS and MALDI TOF MS were developed.[177–179]

6.3. Synergic use of ICP MS and ES MS

The power of the combination of element specific and mass specific information could be demonstrated in a number of studies using model compounds. Synthesized and purified dimethyl-diselenoarsinate salt was analyzed by SEC-ICP AES with the simultaneous arsenic-, selenium-, sulfur-, and carbon-specific detection to reveal an arsenic–selenium moiety with an As : Se molar ratio of 1 : 2.[180,181] ES MS of the chromatographically purified compound showed a molecular mass peak at *m/z* 263 in the negative ion mode. The CID fragmentation of the parent ion produced $(CH_3)_2As^-$ and Se_2^- fragments and enabled the identification of the compound.[180]

Interactions of *cis*-platin with hemoglobin (Hb) were studied using both nanoES-MS and SEC-ICP MS. Size exclusion HPLC separation of free and protein-bound *cis*-platin followed by simultaneous monitoring of ^{195}Pt and ^{57}Fe demonstrated the presence of Hb-bound Pt complexes. NanoES-Q-TOF MS studies of the HbB-*cis*-platin complexes demonstrated further the specific binding of *cis*-platin to the α-chain, heme-α, β-chain, and heme-β units of hemoglobin. Accurate mass measurements and MS/MS information confirmed the existence of HbB-*cis*-platin complexes.[182] A similar methodology was applied to unravel structural changes of hemoglobin that were induced by *cis*-platin binding[183] and interactions of *cis*-platin with rabbit liver MT[184]

The formation and structure of the Au(III)-L-histidine complex was investigated by CE-ICP-MS and MALDI.[185] In a study of the binding of As^{3+} to human MT-2 MALDI-TOF-MS revealed that the structure of the adduct formed by arsenic and hMT-2 was not homogeneous. The maximum molar ratio of As to hMT-2 was found to exceed 6 by ICP-AES.[186] The combined application of SEC-ICP-MS and nanoES MS delivered the complementary element and molecular information required for a reliable characterization of metal-tagged contrast agents of complex, modular design.[152] The quantitative determination of nucleotides from modified DNA was described by reversed-phase HPLC with the parallel ICP-HR MS and ES-MS detection.[187] The combination of flame AAS and ES MS/MS was reported for investigations of speciation of Zn in synthetic solutions containing citrate, oxalate and EDTA.[188]

7. Screening for metal distribution patterns in biological samples: towards speciation analysis

7.1. Size exclusion LC-ICP MS

Several LC-ICP-MS applications exploited the multielement capability of ICP-MS to detect on-line metal binding fractions in biological fluids and cytosols. The preferred chromatographic mechanism was size-exclusion whereas a quadrupole mass analyzer was used in most of the studies. The peak width at half height is about 30 s which allows the simultaneous monitoring of 8–12 isotopes with the precision sufficient for most applications. Recent reports on the multielement ICP Q MS detection in SEC concerned water extracts of soybean flour and white beans,[86] edible mushrooms[189] and nuts.[190] A detailed list of older applications can be found elsewhere.[1]

The use of a sector-field mass analyzer was reported for the detection of protein-bound trace elements in serum[191] and bovine liver.[192] Elemental distribution patterns (15 elements) were measured in whole and skimmed milk and in milk whey,[193] in mussel tissues,[194] and in cancerous and healthy thyroid tissues.[195] SEC was coupled with ICP TOF MS for multisotope speciation (16 isotopes) in carp fish.[196] Advantages and limitations of the different ICP mass spectrometers, employing the quadrupole, double focusing, and TOF mass

analyzers, were discussed with special attention to multi-metal speciation in MTs and MT-like proteins.[197]

A number of worth-mentioning reports used SEC-ICP MS with a single or oligoelement detection. SEC-ICP MS was used to acquire elemental profiles in cytosols of Alzheimer diseased brains.[198] The identification was carried out by means of specific protein assays, *i.e.* enzymatic assays or immunochemical reactions.[39] The fractionation of the SEC separated MT pool revealed that the Cu was associated with a single major MT isoform and bound trace quantities of Zn and Pb.[199] Sulfur analysis by ICP-(DF)MS demonstrated a clear increase of apometallothionein of the major hepatic MT isoform, pointing to its induction in eels under Cd^{2+} exposure.[144] SEC-ICP MS was also reported to study the dynamics of (Cd,Zn)-metallothioneins in gills, liver and kidney of common carp during exposure to cadmium[200] and for speciation of Cd and Pb in cocoa and related products.[201–203]

It should be emphasized that SEC-ICP MS studies are often wrongly referred to as speciation studies. In fact they allow fractionation of the sample only whereas the metal-containing fractions are discriminated from the others by the on-line ICP MS detection. The chromatographic purity of the fractions is usually low and the metal-binding species are usually not identified. Being very robust SEC is well suited to direct injection of complex samples without extensive pretreatment procedure and remains the first chromatographic fractionation step for multidimensional chromatographic approaches.

7.2. Multidimensional chromatography with ICP MS detection

Fractions isolated by SEC can be further fractionated by an independent (orthogonal) separation mechanism with the objective to produce a more detailed map and to achieve a degree of purity of metal species sufficient for their characterization by the molecular MS. Metal complexes with MTs isolated from metal-stressed organisms were widely investigated. Anion-exchange HPLC-ICP MS was the second separation step in studies concerning metal complexes with MT-like proteins in the mussel *Mytillus* at the basal levels,[204] in carp under control and Cd exposure scenarios,[205] and in eel liver.[144] SEC-AE HPLC-ICP MS and stable isotope labeling were used to study the Cu turnover in marine animals.[199] Bidimensional AE-SEC-HR ICP MS allowed the resolution of 7 Ni species in tissues from cancer patients but no difference in binding patterns between cytosols of normal and malignant colon tissues could be revealed.[206] Reversed-phase HPLC-ICP MS was used for the analysis for metal-MT complexes in rat liver and kidney.[207]

Capillary electrophoresis offers a better separation efficiency of MT isoforms and their complexes than HPLC. Capillary electrophoresis-ICP MS was applied to the characterization of the SEC purified fractions in cyanobacterium,[208] rat liver and kidney,[150] human brain[157] and mussel hepatopancreas.[209] The comparability of CZE-ICP-MS electropherograms obtained from different sample matrices during MT separation in human brain cytosols was discussed.[210] CE-ICP MS also allowed the demonstration of the chromatographic purity of the Ni-nicotianamine complex isolated by SEC.[168]

7.3. Gel electrophoresis–laser ablation ICP MS

Gel electrophoresis is attracting attention because of its high resolution. When used for monitoring of metal–protein complexes care needs to be taken to avoid metal loss. Precast Tris-HCl minigels were selected instead of SDS gels for the resolution of cytoplasmic fractions.[139]

A first attempt to detect and characterize multiple metal (Zn, Cd)-binding proteins in complex bacterial (*E. coli*) cytoplasmic fractions.[138] The approach was validated with a well characterized heterogeneously expressed Zn and Cd-binding MT from the cyanobacterium.[138] A direct microlocal technique for protein gel spots was developed for the simultaneous P, S, Si, Cu, Zn and Al in Alzheimer disease brains (*cf.* Fig. 4).[136] It is, however, evident that the resolution of gel electrophoresis for metal complexes will not match that for proteins containing a covalently bound heteroelement.

8. Potential of HPLC-ICP MS for drug metabolism studies

HPLC-ICP MS is a sensitive and robust method for metabolic and pharmacokinetic studies of drugs provided that the target drug and/or its metabolites contain an ICP-detectable element, such as chlorine, bromine, iodine, sulfur, phosphorus or covalently bound metals. ICP MS is becoming a serious competitor to the classically used ES triple quad MS/MS[211,212] because of a higher sensitivity, especially in the presence of a salt-rich biological matrix.

Various platinum-containing drugs were among the most often studied compounds. Studies of Pt anticancer drugs were reviewed.[61] The detection limits at the 0.1 ng mL^{-1} level for Pt in reversed phase[213] and cation-exchange HPLC[214] were reported.

Iodine and bromine are readily ionizable by ICP MS and can be determined without interference with high sensitivity. In this way the metabolic fate of iodinated compounds can be followed without the need for radiolabeled compounds. HPLC-ICP MS with iodine specific detection was applied to the speciation of: 3,3,5,5-tetraiodothyronine (T4), 3,3,5-triiodothyronine (T3), 3,5-diiodothyronine (T2), 3,3,5,5-tetraiodothyroacetic acid, 3,3,5-triiodothyroacetic acid, and 3,5-diiodothyroacetic acid,[115] profiling the iodine-containing metabolites produced by the earthworm *Eisenia veneta* following exposure to 2-fluoro-4-iodoaniline,[215] and the disposition and metabolic fate of 2-, 3- and 4-iodobenzoic acids in rat.[216] Concerning the brominated compounds ICP MS detection was used to follow the metabolism of 4-bromoaniline,[63] bradykinin metabolism in human and rat plasma,[217,218] and 2-bromo-4-trifluoromethylacetanilide in rat urine.[219]

Chlorine-containing drugs were less investigated because of the difficulty of the determination of this element by ICP MS. Nevertheless, a successful use of the ^{35}Cl signal for the quantification of the chlorine-containing drugs diclofenac and chlorpromazine was demonstrated.[62] The simultaneous detection of ^{35}Cl and ^{32}S allowed the quantitative detection of sulfur-containing metabolites such as a sulfate ester and an *N*-acetyl-cysteinyl conjugate.[220] Sulfur-32 was

also demonstrated to be a good tracer for the determination of impurities (at a level below 0.1%) in a drug preparation.[82]

9. The synergy of elemental and molecular MS: on the way to species identification

ICP MS detection was traditionally used for screening biological extracts for the presence of metal-containing fractions which were subsequently analyzed by electrospray MS/MS in search of metal binding-ligands. A typical application of this kind may be the identification of a number of phytochelatins varying in terms of amino acid chain length, amino acid sequence in a Cd-rich fraction detected by on-line ICP MS.[221]

A fundamental question concerns a proof that the metal detected by ICP MS and the ligand identified by molecular MS belong together. The probability of that can be increased by decreasing the chemical complexity of the analyzed fraction. This can be achieved by increasing the resolution of the separation technique or by introducing an additional purification step. For example, the band containing the highest level of Pt isolated by IEF-LA ICP MS of a serum sample was found by using peptide mapping to contain an outer membrane protein which may be involved in *cis*-platin uptake.[139] Similarly, selenium-containing spots in 2D PAGE were demonstrated to contain proteins of which the molecular mass could be determined by ES MS without however a proof that they contained selenium.[222]

The improvement of the purity of heteroatom-containing targets followed by ICP MS also increases the probability of their successful identification by molecular MS. In such cases ICP MS is used only for spotting of heteroelement-containing species and controlling the multidimensional purification processes at the trace level since the final information is obtained uniquely by molecular MS. The inherent discrepancy between the sensitivities of ICP MS and ES MS is partly alleviated by the fact that the metal (detected by ICP MS) constitutes only a small fraction of the whole molecule (detected by ES MS). Combined use of ESI and ICP MS with sulfur specific detection was proposed to measure the relative electrospray ionization efficiency of methionine-containing peptides.[26] Also when the mass spectrometer is used in combination with chromatography the smaller column diameter will favor the detection by electrospray MS which is concentration sensitive in comparison with ICP MS which is a mass sensitive detector.

MALDI is much less vulnerable to matrix suppression than electrospray and produces mostly single charged ions with little fragmentation. These features make it an attractive technique for the identification of target ions for a structural study (amino acid sequencing) by ES MS/MS. Electrospray Q-TOF MS enabled the sequencing of the selenopeptides detected by HPLC-ICP MS.[223]

9.1. Selenium metabolism studies

The essentiality of selenium has stimulated a number of studies on its metabolism in yeast, plants and urine. Speciation of selenium in yeast and plants has been actively researched and was comprehensively reviewed.[52,53] Therefore, here only reports in which the identity of a new compound was demonstrated by tandem MS are discussed. The generic approach was proposed by Casiot *et al.*[224] and was based on the ICP-MS-monitored purification of a selenocompound prior to its identification by ES MS/MS.[224] When applied to yeast it allowed the identification of *Se*-adenosyl-*Se*-homocysteine,[224] *Se*-(methylseleno)cysteine,[225] Se-containing glutathione *S*-conjugates,[226] selenoadenosylmethionine,[227] *Se*-methylselenocysteine, *Se*-homocysteine and *Se*-cystathionine,[228] and a number of *Se*-adenosyl derivatives.[229,230] Also, it allowed the identification of *Se*-methylselenomethionine in *Brassica* roots,[231,232] γ-glutamyl-*Se*-methylselenocysteine in green onions[233] and garlic,[234] a selenium-containing peptide in brazilian nuts,[235] and selenocompounds in Shiitake mushroom.[236] Post-source decay (PSD) was compared with orthogonal quadrupole collision cell dissociation for the purpose of obtaining fragmentation and structural information for ions of non-proteinaceous species ionized by MALDI.[237]

A major urinary metabolite of selenium in rat was identified by HPLC-ICP MS and ESI MS/MS.[56] Its mass spectrum was identical to that of the synthetic selenosugar, 2-acetamide-1,2-deoxy-β-D-glucopyranosyl methylselenide. However, the chromatographic behavior of the Se metabolite was slightly different from that of the synthetic selenosugar and the compound was assigned as a diastereomer of a selenosugar, *Se*-methyl-*N*-acetyl-selenohexosamine (*Se*-methyl-*N*-acetylgalactosamine).[55,56] Using a similar methodology its analogue, in which the methyl group was replaced by glutathion *via* the cysteine sulfhydryl group, was identified.[55]

The presence of *Se*-methyl-*N*-acetylgalactosamine and of a minor metabolite, *Se*-methyl-*N*-acetylglucosamine was confirmed by retention time matching both in reversed-phase and in ion-pairing HPLC-ICP MS.[238] Additionally, the presence of *Se*-methyl-*N*-acetylglucosamine in urine was verified by co-migration with the standard in capillary electrophoresis after fractionation by preparative reversed-phase chromatography.[238] Curiously, trimethylselenonium, selenomethionine, *Se*-methylselenocysteine, *Se*-methylselenomethionine and selenocystamine were not detected in urine samples.[238] This indicates the necessity of caution when interpreting the identification results reported on the basis of retention time matching only.[239] Six Se-containing fractions were isolated from urine by reversed phase HPLC; selenomethionine and selenocystamine were identified in two of them by electrospray MS/MS.[240]

Se-methyl-*N*-acetyl-selenohexosamine was also isolated by solid phase extraction, double reversed-phase followed by SEC but it turned out to be impossible to ionize by electrospray.[241] APCI MS/MS was applied with success instead.[241] Difficulties with efficient ionization of these selenocompounds were also reported by Diaz Huerta *et al.*.[242] *Se*-methylseleno-*N*-acetylglucosamine and *Se*-methylseleno-galactosamine were recently identified in a comprehensive study by nanoES MS/MS.[243]

9.2. Arsenic metabolism studies

Challenges related to the presence of over 20 arsenic compounds in the natural environment and biological systems were highlighted.[244] The study of arsenic metabolism has

benefited enormously from the proliferation of tandem MS used either as a standalone technique or as a detector in HPLC. This technique turned out to be a valuable complement of the classical HPLC-ICP MS approach which had meanwhile become routine. The analytical methodology for arsenic speciation was comprehensively reviewed.[57]

Most of the work targeted the known analytes and resulted in their identity confirmation in addition to the retention time matching.[245–249] Electrospray MS employed in the elemental and in-source fragmentation modes was proposed to detect As species in HPLC.[250] A number of works identified previously unreported compounds by electrospray MS/MS. A new As-containing betaine was identified in fish muscle tissue[251] and novel arsenicals were identified in *Tridacna* clam kidney[252–254] and oyster tissue.[255] The combination of IC-ICP MS and IC-ESI MS/MS was reported for investigation of the chemical stability of arsenosugars in simulated gastric juice[256] and in basic solutions.[257] FT-ICR-MS which offers a high accuracy of the molecular mass measurement was compared with Q-TOF-MS for the characterization of arsenic compounds in complex biological matrices.[258]

Arsenic metabolism in urine was monitored by HPLC-ICP MS with confirmation of the metabolite identity by ES MS/MS. Arsenosugars generated at least 12 As-containing metabolites. A new urinary metabolite dimethylarsinoylethanol was identified.[259] A sulfur containing metabolite of dimethylarsinic acid was detected in rat urine by HPLC-ICP MS and ES MS.[260] A number of dimethylthioarsenical metabolites were detected in rat liver and investigated by ES MS.[261]

Thiol compounds potentially involved in the mechanisms of detoxification of arsenic were investigated. The stability of As-GSH complexes in solution was studied.[59] The identity of the As-induced PCs and of reconstituted As-peptide complexes was confirmed by ES MS. SEC-ICP MS experiments indicated complexation and detoxification of As by the induced PCs.[262] Evidence for the existence of mixed glutathione-phytochelatin-As complex *in vitro* was obtained by the combination of HPLC-ICP MS and electrospray MS.[58] PC_2 was demonstrated by ES MS to be induced by the arsenic exposure in an As hyperaccumulator (chinese brake fern).[263] In another study of the same plant a glutathion PC_2–As complex was identified.[58]

Interaction and binding stoichiometries of trivalent arsenicals with MT were studied. SEC-ICP MS of reaction mixtures between trivalent arsenicals and MT demonstrated the formation of arsenic complexes with MT. Analysis of the complexes using ESI Q TOF revealed the binding stoichiometry between As and the 20 cysteine residues in the MT molecule. Each MT molecule could bind with up to 6 As(III), 10 monomethylarsonous(III) acid, and 20 dimethylarsinous(III) acid molecules which was found to be consistent with the coordination chemistry of these arsenicals.[264]

9.3. Drug metabolism studies

The abilities of ICP MS to provide generic detection for structurally non-correlated compounds with common elements such as P, Br or I and to quantify the molecule based on a signal of the heteroelement gain enormously in value when combined with ES MS/MS used in parallel to confirm the identity of the metabolites detected.[216,217,219,220] In a comparison study between the HPLC-MS/MS and HPLC-ICP MS determinations of a metallodrug in dog plasma samples both methods were shown to be capable of providing accurate and precise results. HPLC-ICPMS offered advantages of extended linear range and 50-times superior sensitivity.[213] The use of HPLC-ICPMS allowed the determination of the relative MS/MS response of the aqua compounds.[213]

HPLC with bromine specific detection was used in combination with ES TOF MS for the investigation of bradykinin metabolism in human and rat plasma[217,218] and for the detection of 2-bromo-4-trifluoromethylacetanilide in rat urine.[219] The natural isotopic doublet of ^{79}Br and ^{81}Br is particularly useful for the spotting of Br-containing metabolites in the corresponding ES-MS spectra.

9.4. Metal complexes with biological ligands

The complementarity of CZE-ICP MS and CZE-ES-MS was evaluated for the characterization of metal complexes with metallothionein.[265] The metal stoichiometry determined by ICP MS matched the molecular weight information obtained for the complexes with the different isoforms by CE-MS. Probing metal-complexes with MTs by reversed phase microbore HPLC and CE coupled with ICP MS and ES MS.[266] Capillary electrophoresis-ES MS/MS was proposed for the characterization of phytochelatins in a Cd-containing fraction isolated by SEC (with ICP MS control of the Cd elution).[267]

ICP-TOF MS and ES-MS were used in parallel in combination with 2D LC (size-exclusion followed by reversed-phase HPLC) for the characterization of metal complexes with MT isoforms in hepatic cytosols of Cd exposed carp. Mass spectra taken at the peak apices indicated the co-elution of different metal (Cd, Cu, Zn and Pb) complexes with MT isoforms within each peak. An in-source protein oxidation procedure was used to identify two MT isoforms.[268]

Citrate was identified by ES-MS-MS in the low molecular weight chromatographic fraction of human serum which had been characterized because of a high Al content detected by AAS.[269] A similar approach using combined SEC-ICP MS and electrospray MS/MS allowed the identification of nicotianamine as a nickel-binding ligand in *Thlaspi c.*[270]

9.5. ICP MS-assisted proteomics

The classical Aanalytical@ proteomic pathway consists in 2D gel electrophoresis, in-gel digestion with trypsin, peptide extraction and a capillary or nanoflow-HPLC-ES MS/MS. This often suffers from the poor sequence coverage as only few fragments are extracted from the gel. In contrast to the identification of the proteins present in the database, where the poor coverage does not preclude a confident result, reliable *de novo* analysis and analysis of covalent modification requires complete sequence coverage. Hence the interest in a reverse approach consisting in digesting a mixture of proteins following the 2D HPLC separation of the peptides formed.

ICP MS can, on one hand, enable the spotting of heteroatom containing proteins in gels scanned by laser ablation and, on the other hand, to detect fractions in LC containing heteroatom-tagged peptides for further characterization. ICP

MS detection can thus limit the number of processed spots or peaks. A considerable advantage of ICP MS over molecular MS is that the information acquired is quantitative. The practical methods reported so far have been scarce and the analytical protocols still need to be refined and extensively validated.

9.5.1. Phosphoproteomics. The most effective approach to detect phoshorylation sites is to trace the fragment ion at *m/z* 79 yielded by the phosphorylated peptides under CID conditions during an LC MS/MS analysis of the digested protein.[35,36] This methodology does not provide information on the number of activated sites. There is no absolute quantification either, except *via* the intensity of the fragment ion for which the CID efficiency can vary with structure. The complementary ICP MS data are particularly valuable in the case of multiple modifications.[137] The determination of phophorus by ICP MS was discussed in Section 4.1.2.

Gel electrophoresis LA-ICP MS was applied to quantify in a mixture a recombinant bovine protein kinase A catalytic subunit, a phosphoprotein completely phosphorylated at four different sites.[119] The P/S ratio (*via* a measured PO^+/SO^+ ratio) in cell cultures was shown to provide a distinguishable difference between malignant cells and primary cultures.[140] An analytical strategy based on 1D PAGE, tryptic digestion, cHPLC-ICP MS and ES MS/MS was proposed for the analysis of human fibrinogen with two well-characterized phosphorylation sites and bovine fetuin with unknown phosphorylation status.[117] Becker *et al.* applied MALDI-FT-ICR MS for the structural identification of phosphorylation in proteins, using direct peptide mapping analysis with high mass accuracy of tryptic phosphorylated fragments.[137]

An alternative strategy based on cHPLC-ICPMS coupling was proposed for the analysis of phosphopeptides[114] in a tryptic digest of β-casein[92] and the identification of the phosphorylation sites in phosphoproteins.[37,118] The complete analytical approach included the sequential application of proteolytic digestion for the generation of phosphopeptides, cHPLC-ICPMS for the determination of their elution time, and cHPLC-MS/MS for the positive identification and sequencing of phosphopeptides.[118] The approach was applied to polo-like kinases. ICP MS provided a quantitative phosphorylation profile of all phosphorylation sites accessible by HPLC.

9.5.2. Selenoproteomics: seleno- and selenium-containing proteins. To date, most information on the presence and distribution of the Se-containing proteins in mammalians was obtained by *in vivo* labeling with ^{75}Se-selenite, separation of the tissue homogenates by SDS-PAGE or 2D electrophoresis and autoradiography of the labeled compounds.[31] Albeit very sensitive, this method has the limitation of the need for radioactive tracers which preclude human studies and the following MS characterization. Selenoproteins and selenium-containing proteins are resistant to denaturation.

Laser ablation ICP MS detection of Se after 1-DE of proteins from a waterfowl embryo and fish-ovary collected from Se-contaminated sites was discussed.[135] A number of bands corresponding to Se-containing proteins were detected. Selenium was enriched distinctly in specific protein bands.[135] A 1D GE-LA ICP MS electropherogram showing a band of glutathione peroxidase from red blood cells.[96] 2D gel electrophoresis was developed to produce reference maps for selenium containing proteins in yeast.[96,222] The detection limit was 0.07 μg g^{-1} gel with single hole drilling and 0.15 μg Se g^{-1} for ablation and translation using ICP DRC MS.[96]

MALDI-TOF MS was predominantly used for the mapping of selenopetides in the enzymatic digests of the purified full length Selenoprotein P and its three smaller isoforms with the identical N termini.[271] *N*- and *O*-glycosylation sites and Se–S and S–S linkages were investigated.[272] Electrospray MS was used for the characterization of the rat mutant selenoprotein W[273] and plant glutathione peroxidase.[274] LC ES MS/MS allowed the unraveling of catalytic intermediates and derivatives of a selenoprotein: phospholipid hydroperoxide glutathione peroxidase.[275]

Oxidation of selenocysteine residue to selenoxide leading to a loss of selenium from selenoproteins during purification by *syn*-β-elimination of selenenic acid was reported.[276] The risk of oxidation of selenocysteine (Se–S and Se–Se bridging) and elimination of dehydroalanine[276] can be prevented by derivatization with iodoacetamide, a protocol well known from gel electrophoresis of cystein containing proteins.[222]

A combination of ICP MS, MALDI TOF MS and electrospray MS/MS was proposed for the identification of a family of Se-containing proteins resulting from the replacement by selenomethionine of 2–9 methionine residues in a salt-stress induced protein SIP18 (M_r 8874). The selenopeptides selected owing to the detector's elemental specificity were then analysed by MALDI-TOF MS in order to select target ions for collision induced dissociation MS. Another selenium protein identified was a heat-shock protein HSP12 (M_r 11 693) in which the only methionine residue was replaced by selenomethionine. The quantification by HPLC-ICP MS accounted for more than 95% of selenium in the water-soluble protein fraction.[277] A linear (low resolution) MALDI spectrum showing the different substitutions was also reported.[277]

10. Outreach

The current advances in hyphenated techniques and cross-fertilization with other "-omics" sectors allow a holistic approach to the metal speciation in individual cells or cell types. A complete fingerprint of the metal-binding components in a cell is likely to provide a new insight into the role of metal ions in biochemistry. To date, effort has been widely dissipated across disciplines, in groups which often do not communicate.

The identification of metals in structural genomics targets is a burgeoning research field which can largely benefit from recent advances in analytical chemistry. Synchrotron radiation sources provide a unique set of structural tools, which in combination can prove extremely powerful in providing a comprehensive picture of these complex biological systems. X-ray absorption spectroscopy was used to examine the selenium biochemistry of *A. bisulcatus*.[278] For metalloproteins, the combined use of X-ray crystallography, X-ray solution scattering and X-ray spectroscopy (XAFS) is extremely useful.[279,280]

The wider use of molecular biology methods is expected to complement the analytical metallomics and lead to an understanding of metalloprotein functions at the molecular level. Validation of metallomic data requires the demonstration of their being linked to the genome and the proteome of a cell. This issue was recently addressed by combining *in vivo* bioanalytical data with *in vitro* molecular genetic data.[270] A complementation screen on a toxic nickel medium allowed the cloning of a gene responsible for metal transport in a hyperaccumulating plant. This allowed the reproduction in yeast of the synthesis of the relevant enzyme (nicotianamine synthase) and the metabolite (nicotianamine) identified earlier *in vivo* by a metallomics approach.[270]

Development of bioinformatics tools provided researchers with the ability to identify full sets of trace element-containing proteins in organisms for which complete genomic sequences are available. Recently, independent bioinformatics methods were used to identify all, or almost all, genes encoding selenocysteine-containing proteins in human, mouse, and *Drosophila* genomes, characterizing entire selenoproteomes in these organisms.[281] It also should be possible to search for entire sets of other trace element-associated proteins, such as metal-containing proteins, although methods for their identification are still in development.[282]

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